

A molecular phylogeny of the subfamily Rogadinae (Hymenoptera: Braconidae) based on the D2 variable region of 28S ribosomal RNA

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Abstract: The D2 variable region of 28S ribosomal RNA was sequenced from ethanol preserved specimens or obtained from the literature to provide the first molecular phylogenetic reconstruction of this subfamily. Evolutionary relationships were investigated by comparing the results using three different analysis methods (distance-based neighbor-joining, NJ; maximum parsimony, MP; and maximum likelihood, ML) and three different outgroups. The monophyly of the Rogadinae *s. s.* is well supported by all trees generated from molecular data. The morphologically defined tribes and the relationships within the subfamily are partially resolved. We found no evidence for a monophyletic Rogadini lineage, neither for the morphological subtribe division within the tribe, but we did find some evidence for three other tribes. Among the genera of Rogadini our results show strong support for the paraphyly of this group. It can be divided into two clades, one consisting of *Aleiodes* + *Arcaleiodes*, the other of *Canalirogas* + *Conspinaria* + *Spinaria* + *Rogas*. The clade *Aleiodes* + *Arcaleiodes* was constantly resolved using different outgroups with all *Aleiodes* species forming a monophyletic group. Two distinct clades are found within *Aleiodes*, strongly corresponding to the morphologically defined subgeneric divisions of the genus. For the clade *Canalirogas* + *Conspinaria* + *Spinaria* + *Rogas*, we found only limited support for this clade to be a monophyletic group. We did not find any support for the genus *Spinaria* to receive subtribe rank. The tribe Clinocentrini is well supported in our analyses, and probably occupy a basal position in the phylogeny of the subfamily. We also found two well-supported clades containing the genera *Yelicones* and *Stiropius*, corresponding to the tribes Yeliconini and Stiropiini, respectively, which were well defined by morphological data, but the placement of these two clades within the phylogeny of the subfamily is poorly resolved in this study.

Key words: Hymenoptera; Braconidae; Rogadinae; 28S rRNA; molecular phylogeny

1 Introduction

The Rogadinae *sensu lato* is a very large subfamily, once including a number of cyclostome tribes that differ widely in biology and share few synapomorphies with one-another (Shaw, 1983; van Achterberg, 1984; Shaw and Huddleston, 1991). It therefore has recently been considered a heterogenous group of polyphyletic (Quicke and Achterberg, 1990; van Achterberg, 1991), or paraphyletic (Shaw and Huddleston, 1991), nature. The concept of Rogadinae *sensu stricto* [= Rogadini of most authors] is widely accepted at present, and defined biologically as all those braconid species that mummify lepidopteran host larvae (Whitfield, 1992; van Achterberg, 1993; Shaw, 1997; Chen and He, 1997). It is

considered to be monophyletic (van Achterberg, 1991) and furthermore was supported by a phylogenetic analysis of relationships in the cyclostome lineages of the Braconidae by Whitfield (1992). However, the phylogenetic relationships among the genera and the evolution of the group in general remain poorly understood. The most current classification scheme for Rogadinae *s. s.* is that of van Achterberg (1993), who divided the subfamily into four tribes, i. e., Clinocentrini van Achterberg, 1991, Rogadini Foerster, 1862, Stiropiini van Achterberg, 1993, and Yeliconini van Achterberg, 1991. Rogadini is the largest of the four tribes and includes the majority of known genera and species; it is subdivided into two subtribes, Spinariina and Rogadina.

Because of its high diversity, estimating a phylogeny of this subfamily is a complex task. To date no molecular phylo-

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genetic study has been concentrated at the generic level in this group.

In this paper we use the 28S gene to resolve the phylogenetic relationships within this group of parasitic wasps. The 28S gene appears to be a promising molecular marker that has been useful for estimating relationships corresponding to generic and subfamily levels of divergence within the family Braconidae (Belshaw and Quicke, 1997a, b; Dowton and Austin, 1998; Mardulyn and Whitfield, 1999).

2 Materials and Methods

2.1 Taxa examined

We examined 28 species belonging to 17 genera. The species and their provenance are listed in Table 1. Identifications of the taxa for gene sequencing were made by the first author. The tribe and subtribe arrangement followed in this paper is that of van Achterberg (1993) and Chen and He (1997) based on morphological and biological characters. Our analysis therefore includes at least one representative of all the four tribes and two subtribes of Rogadini recognized by van Achterberg (1993) and Chen and He (1997).

DNA sequences in this paper have been deposited in EMBL/GenBank/DBJ database under accession numbers listed in Table 1.

2.2 DNA amplification and sequencing

Sequences were obtained either from the literature (see Table 1 for sources) or generated from the taxa that appear in Table 1. Genomic DNA was extracted from insects stored in 100% ethanol. Legs were removed from larger wasps (> 5 mm long) and used for extraction, whereas the abdomen and thorax were used for smaller wasps (< 5 mm long). Ethanol was then removed by washing 3 times (15 min each) in 10 mmol/L Tris-HCl (pH 8.0) containing 100 mmol/L NaCl and 1 mmol/L MgCl₂. Tissue was then ground in 400 μ L of 10 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 1% SDS, and then 100 μ g (or 6 μ L) proteinase K and incubated at 37°C for 1 h. The homogenate was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was redissolved in 100 μ L of sterile water and stored at -20°C.

A portion of the 28S rDNA gene was then amplified. PCR reactions were carried out in 50 μ L volumes containing 100 ng extracted DNA, 5 μ L 10 $\times$ Taq buffer, 4 μ L 25 mmol/L MgCl₂, 4 μ L 2.5 mmol/L dNTPs, 1 μ L 20 μ mol/L primers, 0.5 μ L Taq DNA polymerase (5 U/ μ L). The prim-

ers used for all amplification followed Belshaw and Quicke (1997): 5'-AGA GAG AGT TCA AGA GTA CGT G-3' (forward primer) and 5'-TTG TGC CGT GTT TCA AGA CGG G-3' (reverse primer). PCR conditions were: 30 cycles of 94°C denaturation (30 s), 54°C annealing (1 min), and 72°C extension (1 min) with an initial denaturation of 3 min at 94°C and a final extension for 8 min. PCR products were purified with the QIAquick PCR purification kit and then sequenced directly in both directions using the same primers and TaqFS Dye-labeled terminator on an ABI 377 automated sequencer.

2.3 Sequence alignment

New sequences were deposited in GenBank under accession numbers from AY167645 to AY167659. Sequences were aligned based on distance approach using CLUSTAL X version 1.81 (Thompson *et al.*, 1997) with different gap opening and gap extension values. Because some sequences used in this paper from the literature contain both regions D2 and D3 or other regions (D2 - D10 of *Apis mellifera*), manual alignment was used to remove the D3 or other regions. Lengths of the resulting alignments were between 499 to 560 bp.

2.4 Phylogenetic analysis

Following alignment, three different methods of phylogenetic analysis were performed using PAUP* 4.0 (beta 10 version-Swofford, 2001). First, we used maximum parsimony (MP). To find the most parsimonious tree(s), heuristic parsimony searches (Hillis *et al.*, 1996) were performed using 100 replicates of random addition sequences and the TBR option for branch swapping. Each base was treated as an unordered character with equal weights, with gaps treated as missing data. Where more than one most parsimonious tree was found, a strict consensus tree was calculated. Downweighting transitions or treating gaps as a fifth base did not markedly affect the results obtained. Statistical support for each node was evaluated by bootstrap analysis (Felsenstein, 1985) with 1 000 replicates. Second, we used a distance-based method following the neighbor-joining algorithm (NJ) (Saitou and Nei, 1987) for obtaining a minimum-evolution tree. Bootstrapping evaluation of each node was performed as above. Third, we employed maximum likelihood (ML). ML trees were generated under the HKY85 model using base frequencies estimated by PAUP, and the default number of substitution types (2, HKY85 variant) and transition/transversion ratio (2). Heuristic searches were used with 10 replicates of random addition sequences and TBR branch swapping. Bootstrap analysis was performed with 100 replicates.

Table 1 Taxa examined

Taxa name	Abbreviation in Figs. 1 – 3 (collected locality)	Accession number
Rogadinae		
Clinocentrini		
<i>Clinocentrus politus</i> Chen <i>et</i> He	Clinocenpo (Zhejiang, China)	AY167658
<i>Clinocentrus</i> sp.	Clinocensp	[AJ302890] ⁴
Yeliconini		
<i>Yelicones wui</i> Chen <i>et</i> He	Yeliconesw (Guizhou, China)	AY167659
<i>Yelicones</i> sp.	Yeliconess	[AJ302905] ⁴
Stiropiini		
<i>Stiropius</i> sp.	Stiropiuss	[AJ302904] ⁴
Rogadini		
Spinarina		
<i>Spinaria fuscipennis</i> Brullé	Spinariafu	[Z97964] ²
Rogadina		
<i>Aleiodes aestuosus</i> (Reinhard)	Aleioaestu (Guizhou, China)	AY167655
<i>Aleiodes comexus</i> van Achterberg	Aleiococonve (Guizhou, China)	AY167654
<i>Aleiodes cariniventris</i> (Enderlein)	Aleiocarín (Guizhou, China)	AY167650
<i>Aleiodes miroculatus</i> (Watanabe)	Aleiomiroc (Zhejiang, China)	AY167653
<i>Aleiodes alternator</i> (Nees)	Aleioalter	[AJ302884] ⁴
<i>Aleiodes coxalis</i> (Spinola)	Aleiocoxal	[Z83611] ¹
<i>Aleiodes esenbecki</i> Hartig	Aleioesenb (Shandong, China)	AY167651
<i>Aleiodes gracilipes</i> (Telenga)	Aleiograci (Guizhou, China)	AY167652
<i>Aleiodes mythimae</i> He <i>et</i> Chen	Aleiomythi (Zhejiang, China)	AY167656
<i>Aleiodes similes</i> (Curtis)	Aleiosimil	[AJ302885] ⁴
<i>Arcaleiodes aglaurus</i> (Chen <i>et</i> He)	Arcaleioag (Zhejiang, China)	AY167657
<i>Conspinaria</i> sp.	Conspinari	[AJ302898] ⁴
<i>Rogas</i> sp.	Rogassp	[AJ296058] ³
<i>Canalirogas</i> sp.	Canaliroga	[AJ302889] ⁴
Braconinae		
<i>Amyosoma chinensis</i> (Szépligeti)	Amyosomach (Zhejiang, China)	AY167647
<i>Vipio</i> sp.	Vipiosp (Inner Mongolia, China)	AY167648
Opiinae		
<i>Opius caricivorae</i> Fischer	Opiussp (Zhejiang, China)	AY167649
Histerominae		
<i>Histeromerus mystacinus</i>	Histeromer	[Z83601] ¹
Rhyssalinae		
<i>Rhyssalus</i> sp.	Rhyssaluss	[Z83603] ¹
Ichneumonidae		
<i>Agrypon japonicum</i> Uchida	Agryponjap (Zhejiang, China)	AY167645
<i>Itoplectis naranyae</i> (Ashmead)	Itoplectis (Zhejiang, China)	AY167646
Apidae		
<i>Apis mellifera</i> L.	Apismellif	[AJ302936] ⁴

1. Sequences from Belshaw and Quicke, 1997; 2. Sequences from Belshaw *et al.*, 1998; 3. Sequences from Belshaw *et al.*, 2001; 4. Sequences from Belshaw and Quicke, 2002.

2.5 Outgroup selection

We used 3 different outgroups to analyze tree rooting. The most distant groups were two ichneumonid species (*Agrypon japonicum*, and *Itopectis naranyae*) and one aculeate species (*Apis mellifera*). The Ichneumonidae is widely accepted as the monophyletic sister-taxon to Braconidae both by morphological data (Sharkey and Wahl, 1992; Gauld and Shaw, 1995) and by molecular evidence (Dowton and Austin, 1994), and the aculeate of Hymenoptera is considered as the probable sister-group of the Ichneumonoidea (Rasnitsyn, 1988; Sharkey and Wahl, 1992; Gauld and Shaw, 1995). The second group was the Rhyssalinae and Histeromerinae, two subfamilies of Braconidae that are considered to occupy a basal position within the family (Quicke and van Achterberg, 1990; Whitfield, 1992). The third was the Braconinae (*Amyosoma chinensis*, and *Vipio* sp.) and Opiinae (*Opius caricivora* Fischer), both are relatively close to Rogadinae within the monophyletic cyclostome clade.

3 Results and Discussion

We tested alignments using the Clustal X program with different gap opening and gap extension values, and resulted in different lengths of aligned sequences. The result was identical to that of Morrison and Ellis (1997). They concluded in their paper that the multiple alignments from different procedures varied greatly in length and the alignments produced from the Clustal W program with different gap weights were at least as different from each other as those produced by the different alignment algorithms (Morrison and Ellis, 1997). Because the default parameters in version 1.81 (gap opening 15, gap extension 6.66) were optimized using the balibase multiple alignment benchmark and gave the best overall results in the 142 alignment tests in balibase (J. Thompson, pers. comm.), we used the alignments with default parameters for analysis in the present paper.

The D2 region of 28S rDNA amplified from different taxa ranged from 449 to 482 bp in length; the aligned sequences were different in length when different outgroups were used. For outgroup one, the aligned sequence was 540 bp due to introduction of gaps among sequences. Of 540 total characters analyzed, 224 characters (41.5%) are constant, and 206 characters are parsimony informative (38.2%). Regarding the base composition, the overall CG content is 44.69%,

ranging from 38.16 % to 49.59 %, similar to the results of Belshaw *et al.* (1998): 43 % in Braconidae. The base composition of this region is known to vary widely among other organisms, with CG contents of between 26 % and 85 % recorded (Hancock and Dover, 1988).

The trees resulting from PAUP* analyses are presented in Figs. 1–3. We also show on these figures the bootstrap values obtained from the identical analysis.

From all trees generated from molecular data using different analysis strategies and different taxa as outgroups for comparison, the monophyletic nature of the Rogadinae *s. s.* is well supported, suggesting that the present defined subfamily Rogadinae is a natural group (Figs. 1–3).

The morphological-defined tribes and relationship within the subfamily are partially resolved. We found no evidence for a monophyletic Rogadini lineage, nor for the morphological subtribe division within the tribe, but we did find some evidence for the three other tribes.

Among the genera of Rogadini our results show strong support for a paraphyletic nature of this group. It can be roughly divided into two clades, one consisting of *Aleiodes* + *Arcaleiodes*, the other of *Canalirogas* + *Conspinarina* + *Spinaria* + *Rogas*, both of which may be given tribal rank when more evidence is available to support this branch in the future.

The clade *Aleiodes* + *Arcaleiodes* was constantly resolved using different outgroups (Figs. 1–3), suggesting its monophyletic nature. All *Aleiodes* species also form a monophyletic group with 70–96 bootstrap support. Within *Aleiodes*, two clades are obvious, one containing six species and the other 4 species, with 94–100 and 71–94 bootstrap value support, respectively. This result strongly recovers the morphologically-defined subgeneric divisions of the genus (van Achterberg, 1991; Chen and He, 1997; Belokobylskij and Tobias, 2000). We also found evidence from the molecular data for *Arcaleiodes* Chen and He, 1997 to be a monophyletic and distinct genus (Figs. 1–3).

For the clade *Canalirogas* + *Conspinarina* + *Spinaria* + *Rogas*, we found obvious support for this clade to be a monophyletic group (Figs. 1A, 1B, 2A, 2B, 3A, and 3B, with 64–90 bootstrap support value), but we did not find any support for the genus *Spinaria* to be accorded subtribe rank. The latter was first given tribal rank (van Achterberg, 1988; Quicke and van Achterberg, 1990) due to its remarkably different morphological characters (metasomal tergites form a

carapace), then sunk to subtribal rank (van Achterberg, 1991). The relationships between these genera are still uncertain. We found their phylogenetic relationship as *Rogas* + *Spinaria* + (*Conspinari* + *Canalirogas*) (Fig. 1A, 2B) or *Rogas* + (*Spinaria* + (*Canalirogas* + *Conspinari*)) (Fig. 2A) or *Spinaria* + (*Rogas* + (*Conspinaria* + *Canalirogas*))

(Fig. 1B, 1C) when Ichneumonidae and Apidae, and Rhysalinae and Histeomerinae were used as outgroups, but when the Braconidae and Opiinae were used as outgroups, the relationships became *Canalirogas* + *Rogas* + (*Conspinaria* + *Spinaria*) (Fig. 3A) or *Canalirogas* + (*Rogas* + (*Conspinaria* + *Spinaria*)) (Fig. 3B).

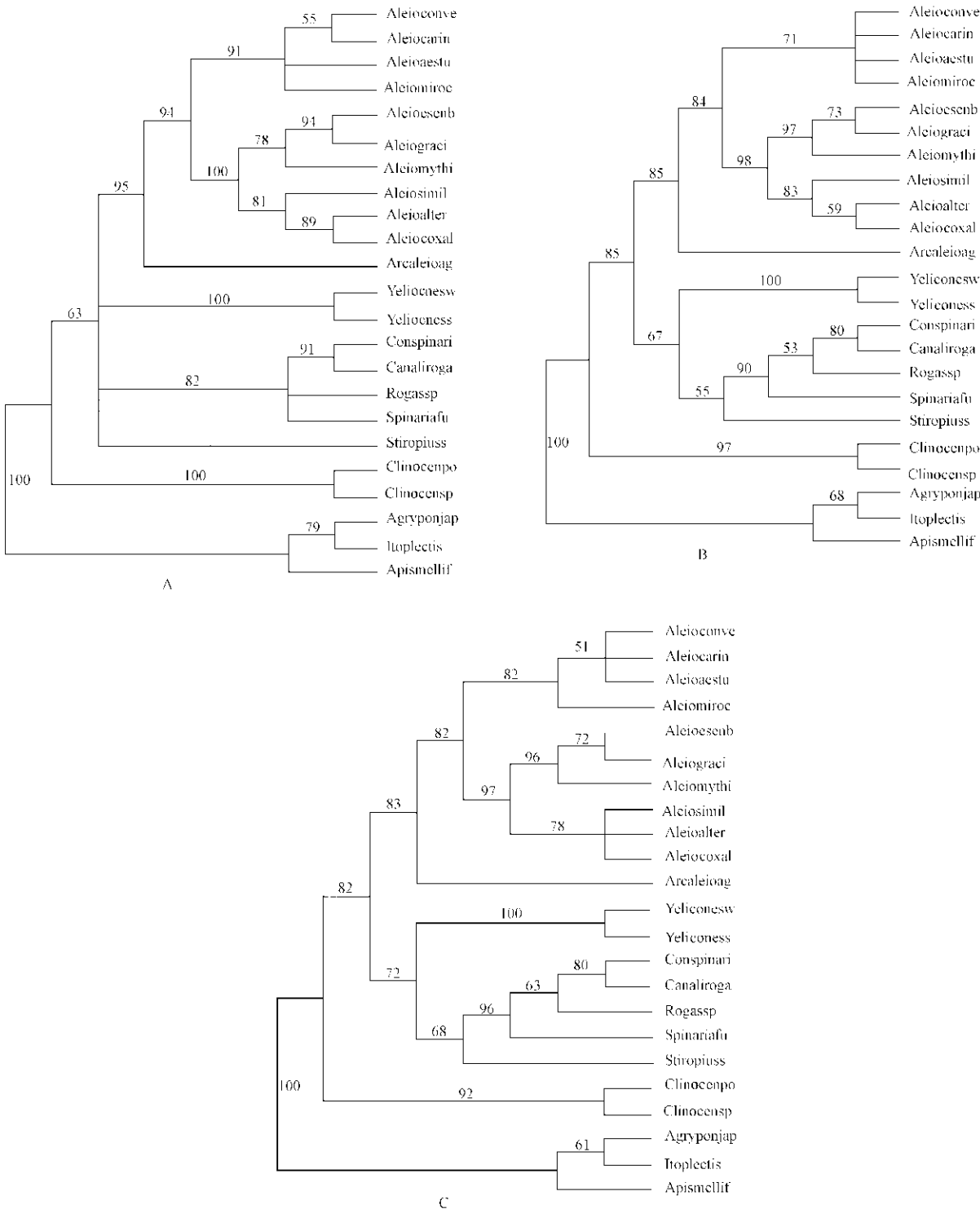


Fig. 1 Phylogenetic analysis using NJ (A), MP (B) and ML (C) methods with Ichneumonidae and Apidae as outgroups
Numbers above branches are bootstrap values (%)

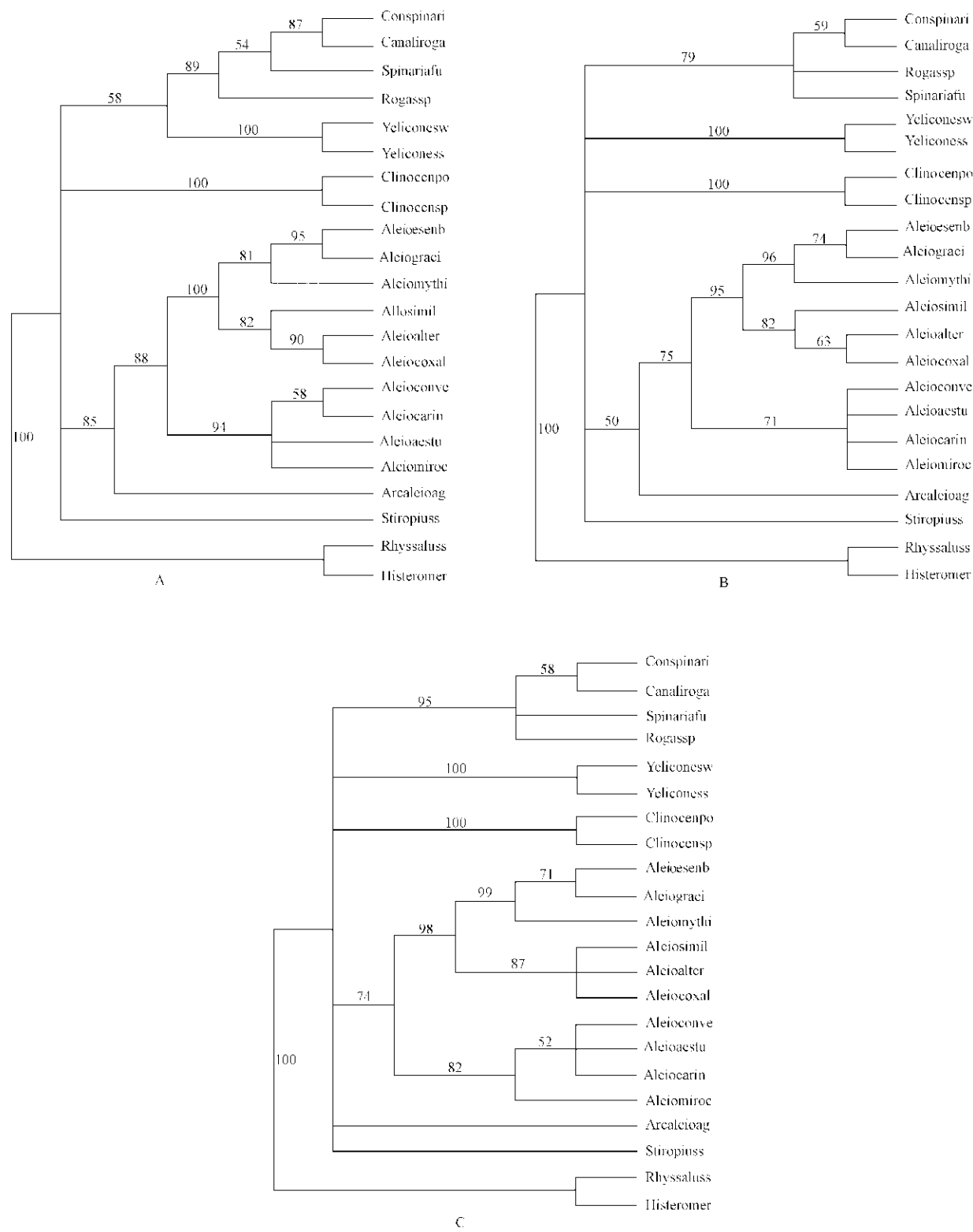


Fig. 2 Phylogenetic analysis using NJ (A), MP (B) and ML (C) methods with Rhyssalinae and Histeromerinae as outgroups
Numbers above branches are bootstrap values (%)

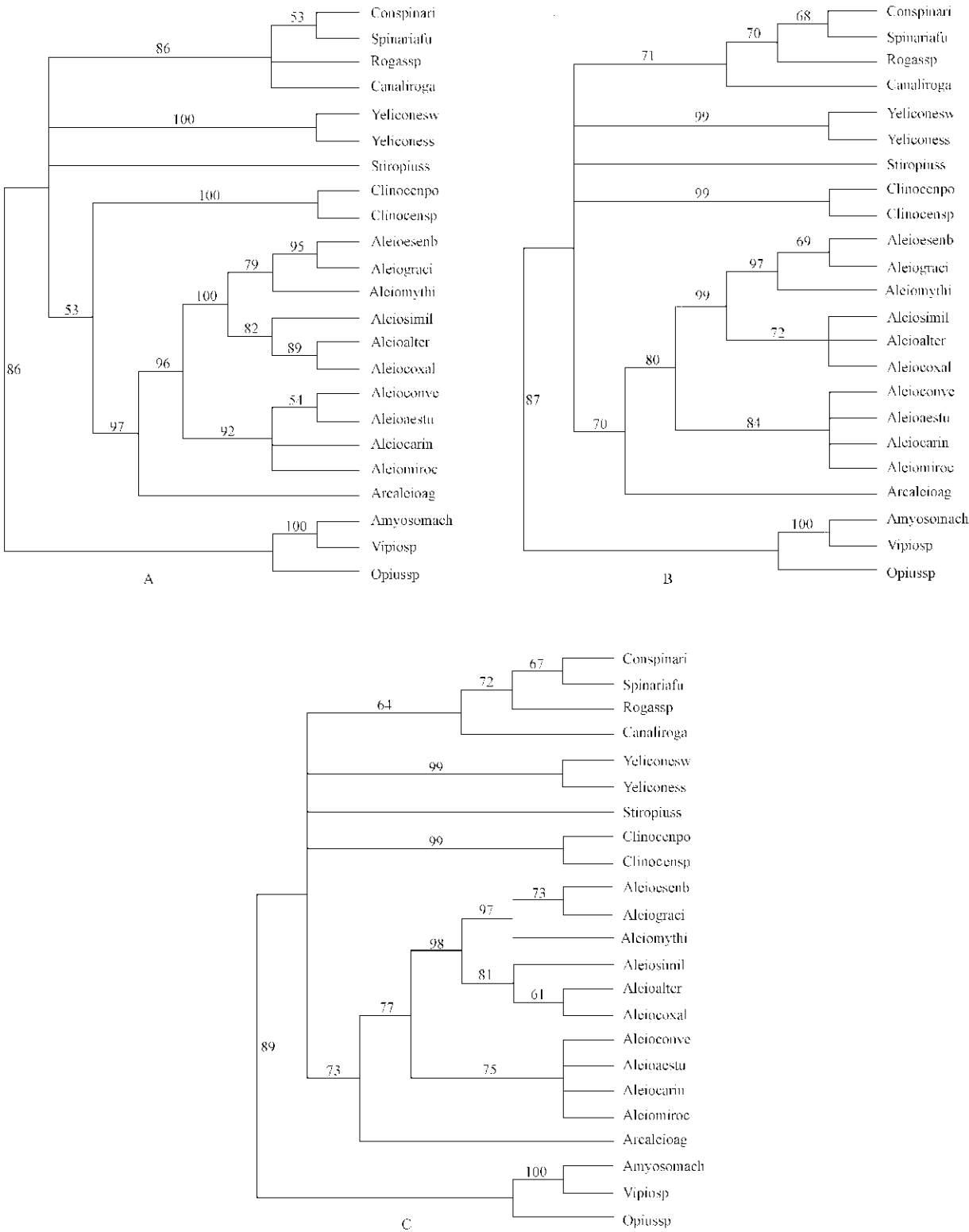


Fig. 3 Phylogenetic analysis using NJ (A), MP (B) and ML (C) methods with Braconinae and Opiinae as outgroups
Numbers above branches are bootstrap values (%)

The phylogenetic relationships of both the clade *Aleiodes* + *Arcaleiodes* and the clade *Canalirogas* + *Conspinaria* + *Spinaria* + *Rogas* with other clades (tribes) within this subfamily are largely unresolved, although the clade *Aleiodes* + *Arcaleiodes* always occupied a terminal position in the phylogeny of the subfamily in most of our analyses (Figs. 1–3).

The tribe Clinocentrini is well supported by our analyses. It occupied a basal position in the phylogeny of the subfamily when Ichneumonidae and Apidae was used as outgroup (Fig. 1: A, B, C), corresponding to the placement of Whitfield (1992). However, the phylogenetic relationships of this clade with other groups within the subfamily are poorly resolved in our other analysis, even becoming the sister-group to the clade *Aleiodes* + *Arcaleiodes* (Fig. 3A), a result never found from morphological characters.

We found a well-supported clade containing the genus *Yelicones*, corresponding to the tribe Yeliconini, which is well-defined by morphological data (a group with the tarsi distinctly shortened). Although the placement of this clade within the phylogeny of the subfamily is poorly resolved, we did find that it was a sister-group to the clade *Rogas* + (*Spinaria* + (*Canalirogas* + *Conspinaria*)) (Fig. 2A) or sister-group to *Stiropius* + (*Canalirogas* + *Conspinaria* + *Spinaria* + *Rogas*) (Figs. 1B, 1C).

The tribe Stiropiini, a morphologically and biologically well-defined group, is also well resolved in our analysis. But its placement within the subfamily is largely unclear, as is its situation with the tribe Yeliconini. It is interesting to point out that in one analysis it was the sister-group of the clade *Canalirogas* + *Conspinaria* + *Spinaria* + *Rogas* (Figs. 1B, 1C), suggesting the similar result presented by Whitfield (1992) that *Stiropius*-group is a sister group of Rogadini s. s. But this group has been hypothesized to occupy a basal position within the phylogeny of the subfamily (van Achterberg, 1991).

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基于 28S rRNA D2 序列的内茧蜂亚科的分子系统发育

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摘要: 首次利用同源 28S rRNA D2 基因序列对内茧蜂亚科 Rogadinae (昆虫纲 Insecta: 膜翅目 Hymenoptera: 茧蜂科 Braconidae) 进行了分子系统学研究。本研究从 95%~100% 乙醇浸渍保存的标本中提取基因组 DNA 并扩增了 10 种内群种类和 5 种外群种类的 28S rDNA D2 片段并测序 (GenBank 序列号 AY167645–AY167659), 利用 BLAST 搜索相关的同源序列, 采用了 GenBank 中 13 个种类的 28S rRNA D2 同源序列, 然后据此进行分子分析。利用 3 个外群 (共 8 个种类) 和 3 种建树方法 (距离邻近法 distance-based neighbor-joining, NJ; 最大简约法 maximum parsimony, MP; 和最大似然法 maximum likelihood, ML) 分析了内茧蜂亚科内的分子系统发育关系。结果表明, 由分子数据产生的不同的分子系统树均显示内茧蜂亚科是一个单系群。内茧蜂亚科内依据形态和生物学特征的分群 (族和亚族) 及其系统发育关系得到部分支持。NJ、MP 和 ML 分析结果均表明内茧蜂族 Rogadini 不是一个单系, 而是一个并系, 其余 3 族则得到不同程度的支持。内茧蜂族可分成 2 个分支: “脊茧蜂属 *Aleiodes* + 弓脉茧蜂属 *Arcaleiodes*” 和 “沟内茧蜂属 *Canalirogas* + 锥齿茧蜂属 *Conspinarina* + 刺茧蜂属 *Spinaria* + 内茧蜂属 *Rogas*”, 二者不是姐妹群。脊茧蜂属 *Aleiodes* 和弓脉茧蜂属 *Arcaleiodes* 始终是姐妹群。脊茧蜂属 *Aleiodes* 是一个单系, 并可分成 2 个姐妹分支, 这与依据形态和生物学特征的亚属分群相一致。弓脉茧蜂属 *Arcaleiodes* Chen *et* He, 1991 是一个独立的属。分支 “沟内茧蜂属 *Canalirogas* + 锥齿茧蜂属 *Conspinarina* + 刺茧蜂属 *Spinaria* + 内茧蜂属 *Rogas*” 的单系性仅得到部分分子数据的支持; 因形态特异 (腹部成甲壳状) 而列为亚族级的刺茧蜂属 *Spinaria*, 分子分析没有证实这一点。横纹茧蜂族 Clinocentrini 是个单系, 并在内茧蜂亚科的系统发育中处于基部 (原始) 的位置。我们研究结果还表明, 阔跗茧蜂属 *Yelicones* 和潜蛾茧蜂属 *Stiropius* 相对应的阔跗茧蜂族 Yeliconini 和潜蛾茧蜂族 Stiropiini 为 2 个独立的分支, 与形态和生物学的结果一致, 但它们在茧蜂亚科的系统发育的位置不明, 有待今后进一步研究。

关键词: 膜翅目; 茧蜂科; 内茧蜂亚科; 28S rRNA; 分子系统发育

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